Phosphate-selective porins from the outer membranes of fluorescent Pseudomonas sp.

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Phosphate starvation induced oligomeric proteins from the outer membranes of Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas aureofaciens, and Pseudomonas chlororaphis were purified to homogeneity. The incorporation of the purified proteins into planar lipid bilayer membranes resulted in stepwise increases in membrane conductance. Single channel conductance experiments demonstrated that these proteins were all capable of forming small channels, similar to the Pseudomonas aeruginosa phosphate porin protein P, with average single channel conductances in 1 M KCl of between 233 and 252 pS. Single channel conductance measurements made in salts of varying cation or anion size indicated that the channels were uniformly anion selective. The measurement of single channel conductance as a function of KCl concentration revealed that all channels saturated at high salt concentrations, consistent with the presence of an anion-binding site in the channel. Apparent $K_d$ values for Cl$^-$ binding were calculated and shown to vary only twofold (180–297 mM) among all channels, including protein P channels. Phosphate competitively inhibited chloride conductance through these channels with apparent $K_i$ values of between 0.59 and 2.5 mM phosphate at 40 mM Cl$^-$. These data were consistent with the presence of a phosphate-binding site in the channels of these phosphate-regulated proteins. Furthermore, they indicated that these channels exhibit at least a 20- to 80-fold higher affinity for phosphate than for chloride.


Des protéines oligomères, induites par privation de phosphates, ont été obtenues des membranes externes de Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas aureofaciens et Pseudomonas chlororaphis, et purifiées jusqu’à l’homogénéité. L’incorporation des protéines purifiées dans la bicouche planaire lipidique des membranes s’est traduite par des augmentations graduelles de la conductance membranaire. Des expériences de conductance par canaux individuels ont démontré que ces protéines étaient toutes capables de former de petits canaux, semblables à ceux produits par la phosphate porine protéine P de Pseudomonas aeruginosa, avec des conductances moyennes par canal dans du KCl 1 M entre 233 et 252 pS. Des mesures de conductance par canaux individuels dans des sels dont les cations et les anions variaient en dimension ont indiqué que les canaux étaient sélectionnés uniformément en fonction des anions. La mesure de conductance d’un canal individuel comme une fonction de la concentration en KCl a révélé que tous les canaux étaient saturés aux plus hautes concentrations, ce qui est en accord avec la présence des sites de liaisons anioniques dans les canaux. Les valeurs $K_d$ apparentes pour les liaisons avec Cl$^-$ ont été calculées et s’avèrent ne varier que du double (180–297 mM) parmi tous les canaux, incluant ceux des protéines P. Les phosphates ont inhibé la conductance des chlorures de façon compétitive à travers ces canaux, avec des valeurs de $K_i$ apparentes entre 0,59 et 2,5 mM de phosphate à 40 mM Cl$^-$ et entre 9,7 et 27 mM de phosphate à 1 M de Cl$^-$. Ces résultats furent concordants avec la présence de liaisons phosphates dans les canaux des protéines régulées par ces phosphates. De plus, ils ont indiqué que ces canaux avaient une affinité plus élevée pour les phosphates que pour les chlorures, d’au moins de 20 à 80 fois.

Introduction

Porin proteins from Gram-negative bacteria form water-filled diffusion channels through the outer membrane which mediate the transmembrane diffusion of hydrophilic molecules below a defined molecular weight cutoff (the exclusion limit) (Nikaido 1979, Benz 1984). Porins, including the phosphate starvation inducible PhoE protein, generally form large (1–2 nm) channels which exhibit only weak ion selectivity in black lipid bilayer membranes (Benz et al. 1983; Benz 1984). In contrast, protein P, a porin protein from Pseudomonas aeruginosa, forms small (0.6 nm), anion-specific channels in reconstituted lipid bilayer membranes (Hancock et al. 1982; Benz et al. 1983).

Protein P is derepressed in wild-type cells of P. aeruginosa under conditions of phosphate deficiency (Hancock et al. 1982). The anion specificity of its channels was demonstrated to be due to an anion-binding site, within the channel (Benz et al. 1983; Benz 1984) formed by positively charged lysine residues (Hancock et al. 1983). Mutational and physiological studies (Hancock et al. 1982; Poole and Hancock 1983, 1984) showed that protein P was coregulated with components of a high-affinity inorganic phosphate transport system in P. aeruginosa, suggesting a role for protein P in this transport system. The recent demonstrations of a phosphate-binding site in the protein P channel (Hancock and Benz 1986) and the altered kinetics of high-affinity phosphate uptake in a mutant deficient in protein P (Poole and Hancock 1986b) strongly support this hypothesis. Phosphate-regulated porins have been identified in other bacteria including members of the families Enterobacteriaceae and Pseudomonadaceae (Overbeek and Lugtenberg 1980; Sterkenburg et al. 1984; Verhoef et al. 1984; Bauer et al. 1985; Poole and Hancock 1986a). Based on their monomer molecular weights and detergent solubilities, most of these phosphate starvation inducible proteins could be grouped with the Escherichia coli PhoEporin (Poole and Hancock 1986a). However, the phosphate starvation inducible porins of the fluorescent pseudomonads, including P. aeruginosa (protein P), P. putida, P. fluorescens, P. aureofaciens, and P. chlororaphis, formed a clearly distinct group on the basis of these properties. To determine whether these proteins also resembled protein P functionally, we purified them and characterized their channel-forming properties in planar lipid bilayer membranes. We report here that these proteins form protein P-like channels in all cases.
Materials and methods

Bacterial strains and media

Pseudomonas aeruginosa (ATCC No. 19305), Pseudomonas putida (ATCC No. 12633), Pseudomonas fluorescens (ATCC No. 949), Pseudomonas chlororaphis (ATCC No. 9446), and Pseudomonas aureofaciens (ATCC No. 13985) were the strains employed in this study. With the exception of P. fluorescens, all strains were type strains.

The sodium Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffered phosphatase-sufficient and phosphatase-deficient minimal media have been described previously (Hancock et al. 1982). Strains were routinely maintained on L agar medium.

Preparation of a protein P trimer specific antiserum (200 μL) was stored at 4°C.

Protein P was purified as described previously (Nicas and Hancock 1980). Solubilized cell envelopes were prepared by extracting once with 2% (w/v) Triton X-100, pH 8.0 - 10 mM ethylenediaminetetraacetate (EDTA), pH 8.0, after 30 min incubation at 37°C in the presence of 1 mg lysozyme/mL as described (Hancock et al. 1981). Sodium dodecyl sulphate (SDS) – polyacrylamide gel electrophoresis (PAGE) was performed as described previously (Hancock and Carey 1979) using a 12% (w/v) acrylamide running gel.

Preparation of a protein P – Affi-gel-10 column

Protein P, purified as described previously (Hancock et al. 1982), was passed across a Biogel P-10 column equilibrated with 0.1 M acetic acid, 2% (w/v) yeast extract, and 0.05% (w/v) NaCl - 2% (w/v) Bactoagar and phosphate-sufficient minimal medium plates (phosphate-sufficient medium + 2% (w/v) Bactoagar).

Preparation of antisera and construction of an anti-protein P immunoadsorbent column

Antiserum specific for protein P trimers was prepared as described previously (Poole and Hancock 1986b). Protein P trimmer specific antibodies were purified free of serum proteins by incubating the protein P trimer specific antisera (200 μL) on the above protein P–Affi-gel-10 affinity column (1.5 mg) was cross-linked to approximately 0.65 mL of Affi-gel-10 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) for 2 h at room temperature as recommended by the manufacturer. The column was stored at 4°C in phosphate-buffered saline (PBS), pH 7.4 (Mutharia and Hancock 1983) containing 0.1% (w/v) Triton X-100. Prior to use, the column was washed exhaustively with PBS to remove excess detergent.

Preparation of phosphate starvation induced outer membrane proteins

Two methods were used for the purification of phosphate starvation induced outer membrane proteins. The first was an affinity chromatography method utilizing an immunoadsorbent column. Triton X-100 insoluble cell envelopes prepared from 100 mL stationary phase cultures of phosphate-limited cells were solubilized in 1 mL 2% (w/v) Triton X-100 - 20 mM Tris–HCl, pH 8.0 - 10 mM EDTA containing 1 mg lysozyme/mL at 37°C for 30 min. The Triton X-100 – EDTA – lysozyme soluble fractions (300 μL) were subsequently incubated on the anti-protein P immunoadsorbent column at room temperature. After 45 min incubation, the column was washed successively with 3 mL of 2% Triton X-100 – 20 mM Tris–HCl, pH 8.0, and 3 mL of 2% (w/v) Triton X-100 – 20 mM Tris–HCl, pH 8.0 - 0.5 M NaCl to remove unbound and nonspecifically bound material, respectively. Material bound specifically to the column was eluted with 3 mL of 0.1 M glycine–HCl, pH 2.5, and fractions collected in tubes containing solid Tris base as described above. Peak fractions, as determined by SDS–PAGE, were pooled.

The second method involved electroelution of proteins from SDS–polyacrylamide gels. Triton X-100 – EDTA – lysozyme soluble cell envelopes (described above) (300 μL) were solubilized at room temperature in an equal volume of solution mix without 2-mercaptoethanol (Hancock and Carey 1979), loaded onto individual SDS–polyacrylamide slab gels (1.5 mm thickness), and electrophoresed to separate protein components. Phosphate starvation induced membrane protein oligomer bands were subsequently excised and the proteins extracted from the gel via electroelution as described previously (Parr et al. 1986). The eluted proteins (5 mL, final volume) were concentrated 10-fold against solid polyethylene glycol (20000 molecular weight, Sigma Chemical Co., St. Louis, MO, U.S.A.) before being dialysed at room temperature for 16 h against 1 L of 0.1% (w/v) Triton X-100 – 20 mM Tris–HCl, pH 8.0, with one buffer change.

Black lipid bilayer experiments

The methods used for black lipid bilayer experiments have been described previously in detail (Benz et al. 1979; Benz and Hancock 1981). The apparatus consisted of a Teflon chamber with two compartments separated by a small hole (0.1 mm² for single channel experiments; 2 mm² for macroscopic conductance experiments). A membrane was formed across the hole by painting on a solution of 1.5% (w/v) oxidized cholesterol in n-decane. Bilayer formation was indicated by the membrane turning optically black to incident light. In single channel conductance experiments, conductance through the pores was measured after application of a given voltage using a pair of Ag/AgCl electrodes inserted into the aqueous solutions on both sides of the membrane. The current through the pores was boosted by a preamplifier, monitored by a storage oscilloscope, and recorded on a strip chart recorder.

The procedure for macroscopic conductance inhibition experiments has been described (Hancock and Benz 1986). Briefly, the Ag/AgCl electrodes were replaced with Calomel electrodes and the current through the pores was monitored with a Keithley 610B electrometer. After addition of purified protein P to the solutions bathing the lipid bilayer membrane, the increase in conductance (measured as current increase) was followed for 15–25 min or until the rate of increase had slowed considerably. At this time membrane conductance had increased 2–4 orders of magnitude. The bathing solutions in both compartments were stirred gently (approximately 60 revolutions per minute) with a magnetic stir bar and aliquots of phosphate added to both compartments. Sufficient time (30–90 s) was allowed for the new current level to be established before addition of subsequent aliquots.

Results

Purification of the phosphate starvation inducible outer membrane proteins of the fluorescent pseudomonads

Members of the Pseudomonas fluorescens branch of the family Pseudomonadaceae, including P. putida, P. fluorescens, P. aureofaciens, and P. chlororaphis, were previously demonstrated to synthesize oligomeric, heat-modifiable outer membrane proteins which exhibited a number of properties in common with protein P of P. aeruginosa (Poole and Hancock 1986a). Using the observed-immunological cross-reactivity of these proteins with protein P, an attempt was made to purify these phosphate-regulated proteins by specific retention on an
immunoadsorbent column constructed with the protein P trimer specific antiserum described previously (Poole and Hancock 1986b). Protein P, the original antigen, was readily purified in reasonable quantities by this method (Fig. 1, lanes 1 and 2). It was also possible to isolate the other phosphate starvation inducible outer membrane proteins using this column, although the yields were substantially lower, requiring a sensitive silver staining procedure to detect the proteins on Coomassie stained gels. Again, the purified oligomer bands excised, and the protein was electroeluted from the gel. This method produced substantially increased yields of all proteins (Fig. 1, lanes 5–12) which were easily visible on Coomassie stained gels. Again, the purified proteins retained their oligomeric structure, as attested by their resistance to SDS denaturation (Fig. 1, lanes 6, 8, 10, and 12), unless heated at high temperature (Fig. 1, lanes 5, 7, 9, and 11).

**Single channel experiments**

When the purified phosphate starvation inducible outer membrane proteins were added in small quantities (5–10 ng/mL) to the aqueous solutions bathing a black lipid bilayer membrane, membrane conductance was seen to increase in a stepwise fashion (e.g., Fig. 2), presumably due to the incorporation of individual protein oligomers into the membrane, as suggested for other porins (Benz et al. 1979; Benz and Hancock 1981). Most of the observed single channel conductance increments were distributed about a mean (e.g., Fig. 3, $\Delta_1$), although larger increments were also seen at $2 (\text{Fig. } 3, \Delta_2)$, 3 and 4 (not shown) times the average single channel conductance. These larger increments probably represented aggregates...
TABLE 1. Channel-forming properties of affinity-purified and electroeluted phosphate starvation inducible outer membrane oligomers of the fluorescent pseudomonads

<table>
<thead>
<tr>
<th>Source of phosphate starvation inducible outer membrane protein</th>
<th>Affinity purified</th>
<th>Electroeluted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single channel conductance (pS)</td>
<td>n</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>239</td>
<td>317</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>233</td>
<td>74</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>241</td>
<td>117</td>
</tr>
<tr>
<td><em>P. aureofaciens</em></td>
<td>237</td>
<td>54</td>
</tr>
<tr>
<td><em>P. chlororaphis</em></td>
<td>243</td>
<td>45</td>
</tr>
</tbody>
</table>

*Average value for n events. The probability histograms of single channel conductances (Fig. 3) were overlapping for all proteins.

Table 2. Single channel conductance of phosphate starvation inducible porin proteins of the fluorescent pseudomonads in salts of varying anion and cation size

<table>
<thead>
<tr>
<th>Source of porin</th>
<th>K⁺Cl⁻</th>
<th>Tris⁺Cl⁻</th>
<th>K⁺Hepes⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>158</td>
<td>141</td>
<td>nd</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>144</td>
<td>143</td>
<td>nd</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>156</td>
<td>149</td>
<td>nd</td>
</tr>
<tr>
<td><em>P. aureofaciens</em></td>
<td>164</td>
<td>169</td>
<td>nd</td>
</tr>
<tr>
<td><em>P. chlororaphis</em></td>
<td>166</td>
<td>167</td>
<td>nd</td>
</tr>
</tbody>
</table>

*Average of at least 60 single channel events. Salts were employed at a concentration of 0.5 M at pH 6.0. Ion radii (in nm) are as follows: K⁺, 0.133; Cl⁻, 0.181; Tris⁺, 0.670. Hepes⁻, an ellipsoid molecule, has the dimensions 1.4 × 0.6 × 0.5 nm. *No conductance increments were resolved. The minimum resolvable conductance increment in the above experiment was 10 pS, although the single channel conductance of protein P channels in K⁺Hepes⁻ was previously found to be <2 pS (Hancock et al. 1982).*

or simultaneous insertions of two or more protein oligomers into the bilayer membrane (Hancock et al. 1982). The average single channel conductances in 1 M KCl, measured for a given protein purified by either affinity chromatography or electroelution, were very similar (Table 1), confirming both the utility of the anti-protein P immunoabsorbent column in purifying functional cross-reactive molecules and the general applicability of the electroelution procedure in purifying functionally active porin proteins. The average single channel conductance values obtained for the individual phosphate starvation inducible proteins were also very similar to one another, falling between 233 and 252 pS (Table 1). These values were substantially less than those obtained for the phosphate starvation inducible PhoE porins of *E. coli* and *Salmonella typhimurium* (approximately 2 nS (Benz et al. 1983; Bauer et al. 1985)). They were, however, in excellent agreement with the observed single channel conductance of protein P in 1 M KCl (Table 1).

**Ion selectivity**

To probe the ion selectivity of these channels, single channel conductance was measured in the presence of salts of varying cation or anion size. The anion-specific protein P channel has been previously demonstrated to yield average single channel conductances, the magnitudes of which were dependent exclusively upon the size of the anion (Benz et al. 1983). Thus, conductance through protein P channels was demonstrated to be inversely related to the size of the anion (Benz et al. 1983), while remaining basically unaffected by changes in cation size of the salt bathing a protein P containing lipid bilayer membrane (Hancock et al. 1982). The results in Table 2 demonstrated that in all cases the observed single channel conductance was dependent upon anion size only, such that increasing the anion size in the case of K⁺Hepes⁻ (anion dimensions of 1.4 × 0.6 × 0.5 nm compared with an anion radius of 0.181 nm for K⁺Cl⁻) resulted in no detectable conductance increments, while increasing the cation size in the case of Tris⁺Cl⁻ (cation radius of 0.67 nm compared with 0.133 nm for K⁺Cl⁻) yielded a single channel conductance which was not substantially different from that observed in K⁺Cl⁻. These data were consistent with the formation of anion-selective, if not specific, channels by these proteins.

The anion specificity of protein P has been shown to be due to the presence of an anion-binding site within the channel (Benz et al. 1983). Thus conductance through protein P channels saturates at higher salt concentrations (Benz et al. 1983), in contrast to other porins, including PhoE, which lack binding sites and typically reveal a linear dependence of single channel conductance on salt concentration (Benz and Hancock 1981; Benz et al. 1984). To determine if the anion selectivity of the phosphate-regulated porin proteins could be attributed to binding sites within their respective channels, single channel conductance was measured as a function of salt (KCl) concentration. In every case, single channel conductance was seen to saturate at higher salt concentrations (e.g., Fig. 4) consistent with the presence of a binding site within these channels. By
replotting the data as an Eadie–Hofstee plot (e.g., Fig. 4, inset), $K_a$ values were obtained by least squares analysis.

\*Inhibition of macroscopic chloride conductance by phosphate was carried out as described in Materials and methods. The percent inhibition of initial conductance was measured for different concentrations of phosphate and the data plotted as an Eadie–Hofstee plot (see Fig. 4, inset) from which $I_{50}$ values (defined as the concentration of phosphate yielding 50% inhibition of chloride conductance) were obtained by least squares analysis of three independent experiments.

It has been demonstrated, for certain porin proteins, that the rate of porin incorporation into black lipid bilayer membranes decreases drastically at low concentrations of salt in the aqueous phase (R. E. W. Hancock, unpublished observation). At the levels of porin protein available, the numbers of porin channels inserting into the membrane at 40 mM KCl were insufficient to permit the accurate measurement of phosphate inhibition of chloride conductance, which usually required approximately 1000 channels per membrane protein P.

### Phosphate inhibition of macroscopic conductance

The presence of a phosphate-binding site within *P. aeruginosa* protein P channels has been previously confirmed by the ability of orthophosphate to inhibit Cl\(^{-}\) conductance through protein P channels (Hancock and Benz 1986). To identify potential phosphate-binding sites in the phosphate starvation induced porin proteins of *P. putida*, *P. fluorescens*, *P. aureofaciens*, and *P. chloraphis*, this strategy was also applied in this study. Thus after formation of a lipid bilayer, a small amount of protein was added to the aqueous solution bathing the lipid membrane and conductance followed until the rate of increase had slowed considerably (usually 15–25 min).

At this time membrane conductance had usually increased by 2–4 orders of magnitude and >1000 channels were present in the membrane. Aliquots of phosphate were added sequentially and the new conductance level measured after each addition.

For each protein studied, phosphate addition was seen to decrease the level of conductance originally observed in the presence of KCl alone, and the magnitude of this decrease was directly related to the concentration of phosphate added (e.g., Fig. 5). By plotting the data as percent inhibition of chloride conductance as a function of percent inhibition of chloride conductance – phosphate concentration (i.e., an Eadie–Hofstee plot) (Fig. 5, inset), it was possible to derive an apparent $I_{50}$ value for phosphate inhibition of chloride conductance for each of the phosphate-regulated porins (Table 3). These data were consistent with the presence of a phosphate-binding site within each of these channels. The apparent $I_{50}$ values varied with the concentration of KCl, ranging from 9.7 to 27 mM phosphate in 1 M KCl, and from 0.59 to 2.5 mM phosphate in 40 mM KCl (at pH 7) (Table 3). At a given concentration of KCl, however, the variation in $I_{50}$ values obtained for all of the channels did not exceed fourfold, indicating that the relative affinities of these channels for phosphate were quite similar.

### Discussion

It was previously suggested (Poole and Hancock 1986a) that phosphate starvation inducible outer membrane proteins expressed by members of the families *Pseudomonadaceae* and *Enterobacteriaceae* could be grouped into two classes based on monomer molecular weights and the detergent conditions necessary to release the proteins from the peptidoglycan. The best-studied representatives of these two classes are the *E. coli* PhoE protein and *P. aeruginosa* protein P, respectively. These two porins are also distinguishable from a functional viewpoint. Thus, while the PhoE protein forms large (1 nm), weakly anion selective channels (Darveau et al. 1984; Benz et al. 1985) which lack strong binding sites for anions or phosphate (Benz et al. 1984), protein P channels are small (0.6 nm) (Hancock et al. 1982), and possess binding sites for anions and phosphate (Hancock and Benz 1986; Table 3) consistent with the observed anion specificity and phosphate selectivity of this channel (Hancock and Benz 1986; Table 3). The demonstration here that those proteins resembling protein P in molecular weight and detergent solubility also formed anion-specific – phosphate-
selective channels similar to that of protein P (Tables 2 and 3) supports the existence of these two classes, as does the observation that those enterobacterial phosphate-regulated porin proteins which have been examined (Verhoef et al. 1984; Bauer et al. 1985) form larger, less selective, PhoE-like channels.

Based on the results of DNA and rRNA hybridization studies, the strains examined here have been classified in the same homology group (the Pseudomonas fluorescens group) and are clearly distinct from other species of the family Pseudomonadaceae (Palleroni et al. 1973). Despite differences in the biochemical properties of those strains which produce a protein P-like, phosphate starvation inducible porin, they appear to have additional, quite similar, outer membrane proteins. Each of these fluorescent pseudomonads produces a lipoprotein which cross-reacts immunologically with outer membrane protein H2 of P. aeruginosa PAO1 (Mutharia and Hancock 1985). In addition, all of these strains produce a major, constitutive outer membrane protein, which resembles porin protein F both in molecular weight and inability to form SDS-stable oligomers in polyacrylamide gels (Mutharia and Hancock 1983; K. Poole, unpublished data). In contrast, members of the Enterobacteriaceae and other pseudomonads, including P. cepacia and P. pseudomallei, each produce a major, constitutive outer membrane protein which forms native oligomers which are resistant to denaturation by SDS at temperatures below 60°C (Lugtenberg and van Alphen 1983; K. Poole, unpublished data). Thus, these data are consistent with the existence of an evolutionary–taxonomic link between the fluorescent Pseudomonas sp. and emphasize the potential importance of outer membrane protein profiles and antigenic similarities amongst surface proteins as indicators of such links.

The existence of a protein P-like porin in a number of distinct bacterial species affords the opportunity to perform comparative studies on the biogenesis of these proteins and to identify their functional domains. Given the similarities of the functional properties of these proteins, which were quite distinct from other phosphate-regulated porins, it should be possible to identify regions of the protein involved in, for example, anion–phosphate selectivity as regions of close homology in the genes encoding these proteins. Such data will undoubtedly contribute to our goal elucidating the topology of protein P in P. aeruginosa.

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